The Enzymatic Synthesis of Thiamine Monophosphate
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The catalysis, by the enzyme thiaminase, of reversible base-exchange reactions between thiamine and aromatic tertiary amines was first reported by Fujita et al. (1). Related findings with mammalian dihydrophosphopyridine nucleotidases (2) and with methionine activation (3) drew attention to the energy-rich nature of "onium" (4-6) compounds and suggested that the alkylation of thiazole must involve activation of the pyrimidine, 2-methyl-4-amino-5-hydroxymethylpyrimidine. That the pyrimidine might be activated by phosphorylation was suggested by the observation of Harris and Yavit (7) that in crude extracts of bakers' yeast, the phosphate ester of the pyrimidine might be a precursor of thiamine than the pyrimidine compound. In independent studies, Leder (8) and Nose, Ueda, and Kawasaki (9), using synthetic substrates, showed that the products were the pyrophosphate ester of the pyrimidine and a phosphorylated thiazole, and that the initial reaction product was not thiamine, but a phosphorylated form of thiamine not previously identified (10, 11) as thiamine monophosphate. Caminer and Brown also showed (12) that 2-methyl-4-amino-5-hydroxymethyl pyrimidine monophosphate and a second pyrimidine derivative, identified on presumptive evidence, as the corresponding phosphorylated thiazole, were formed enzymatically in partially purified yeast preparations from 2-methyl-4-amino-5-hydroxymethyl pyrimidine and adenosine triphosphate. Evidence for the sequential formation of these esters by separate enzymes has been reported by Lewin and Brown (13). The results of these studies may be summarized by the following equations:

\[
\begin{align*}
\text{Hydroxymethylpyrimidine} + \text{ATP} & \rightarrow \text{hydroxymethylpyrimidine-P} + \text{ADP} \\
\text{Hydroxymethylpyrimidine-P} + \text{ATP} & \rightarrow \text{hydroxymethylpyrimidine-PP} + \text{ADP} \\
\text{Thiazole} + \text{ATP} & \rightarrow \text{thiazole-P} + \text{ADP} \\
\text{Hydroxymethylpyrimidine PP} + \text{thiazole P} & \rightarrow \text{thiamine-P} + \text{PP}
\end{align*}
\]

In this paper, the synthesis of the phosphorylated pyrimidines and the purification and properties of the enzyme that catalyzes reaction (4) are described. The name, thiamine-P pyrophosphorylase, is proposed for this enzyme.

* The abbreviations used are: hydroxymethylpyrimidine, 2-methyl-4-amino-5-hydroxymethylpyrimidine; thiazole, 4-methyl-5-(β-hydroxethyl) thiazole; DEAE-cellulose, N,N-diethylaminoethyl cellulose.

EXPERIMENTAL PROCEDURE

Methods

Thiamine and thiamine phosphate were determined fluorimetrically by oxidation to the corresponding thiochromes with ferricyanide (14) or cyanogen bromide (15), the fluorescence generally being measured directly without extraction into n-hexanol or isobutanol. The CNBr procedure was modified as follows. A sample containing 0.01 to 0.2 mpmole of thiamine in 0.5 ml was acidified by the addition of 0.025 ml of 2.5 n HCl. In enzymatic studies, this stopped the reaction. This was followed in turn, with mixing after each addition, by 0.3 ml of CNBr (prepared by decolorizing ice-cold saturated bromine water with 10% KCN) and 0.2 ml of 30% sodium hydroxide. The fluorescence was measured in a Farrand photofluorometer. Stable levels of fluorescence are not obtained by the CNBr procedure if samples are acidified with trichloroacetic acid in place of hydrochloric acid.

Thiamine-P could be distinguished from thiamine by extracting the thiochrome reaction mixture with n-hexanol. Under these conditions, thiochrome, but not thiochrome-P, is extracted. Phosphate was determined by the method of Fiske and SubbaRow (16) and by the sensitive method of Chen (17). For total phosphate, ashing was carried out with magnesia nitrate as described by Ames and Dubin (18).

Materials

Thiamine-P and protamine sulfate were obtained from Nutritional Biochemicals Corporation and thiamine-PP from Schwarz BioResearch, Inc. 2-Methyl-4-amino-5-bromomethylpyrimidine dihydrobromide and 4-methyl-5-(β-hydroxyethyl) thiazole were kindly supplied by Merck and Company, Inc. Thiazole-P and thiazole-PP were obtained by bisulfite cleavage of the corresponding thiamine esters (19).

Hydroxymethylpyrimidine was prepared from the corresponding bromide dihydrobromide by treating an aqueous solution with 3 equivalents of silver nitrate. The silver bromide was removed by filtration, washed with water, and the combined filtrates were made alkaline with sodium hydroxide. The hydroxymethylpyrimidine was obtained in 80 to 90% yield by evaporating to a small volume under reduced pressure. The compound has a marked positive temperature solubility coefficient and was readily recrystallized from water.

Hydroxymethylpyrimidine-P and hydroxymethylpyrimidine-PP were prepared as follows: Hydroxymethylpyrimidine hydrochloride, 1 g, was added in several small portions to 8 g of pyro-
phosphoric acid at approximately 70°, and the mixture stirred at 105-110° for 20 minutes. The syrup was poured into 100 ml of rapidly stirred ethanol at -15° followed by the addition of 300 ml of ether. The flocculent precipitate was collected by centrifugation, washed twice with ether and dried over silica gel under reduced pressure. The white hygroscopic solid, weighing approximately 1.5 g, was dissolved in cold water, adjusted to pH 6.5, and poured on to a column (2 x 18 cm) of Dowex 50-X8 H+, and the pyridimidine compounds were eluted with water. Inorganic phosphate and hydroxymethylpyrimidine-PP appeared immediately after the hold-up volume. Hydroxymethylpyrimidine-P was eluted after approximately 8 bed-volumes of water had run through the column and was obtained as a white solid weighing 300 mg after evaporating the eluate (600 ml) to a small volume under reduced pressure. Hydroxymethylpyrimidine-P was also prepared by the method of Makino and Koike (20).

The hydroxymethylpyrimidine-PP solution, adjusted to pH 7.0, was adsorbed on a 3- x 10-cm column of Dowex 1-formate and recovered by gradient elution with ammonium formate with a mixing volume of 500 ml and approaching 1 m ammonium formate. Elution was followed by observing ultraviolet quenching by spots applied to paper. After most of the ammonium formate was removed by lyophilization, the hydroxymethylpyrimidine-PP was isolated as the barium salt, weighing 340 mg.

Properties of Pyrimidine Phosphates—The ultraviolet absorption spectra of hydroxymethylpyrimidine-P and hydroxymethylpyrimidine-PP were identical with that of hydroxymethylpyrimidine, each having a single maximum at 246 mp in 0.1 N HCl. Preparations of both compounds were free of orthophosphate and, on the basis of the molar extinction coefficient of approximately 11.2 x 10^4 cm^2 per mole, contained the requisite molar ratio of organic phosphate (Table I). It is of particular interest that the phosphate groups of both pyrimidines are readily hydrolyzed by acid. Fig. 1 shows that the rate of hydrolysis of the two phosphate groups of hydroxymethylpyrimidine-PP by 1 N H_2SO_4 at 100° is almost as rapid as that of the acid-labile phosphate groups of ATP. Hydroxymethylpyrimidine-P is hydrolyzed at a slower rate, but is completely hydrolyzed by 2 N sulfuric or hydrochloric acid at 100° in approximately 20 minutes. Hydrolysis is somewhat slower in 1 N acid; 30 and 77% in 10 and 20 minutes for hydroxymethylpyrimidine-P and 60 and 90% for hydroxymethylpyrimidine-PP under the same conditions. These results are somewhat at variance with the report of Camiener and Brown (11), who characterized hydroxymethylpyrimidine-P as acid-stable and hydroxymethylpyrimidine-PP acid-labile with respect to hydrolysis to hydroxymethylpyrimidine by 1 N HCl. Under relatively mild conditions, 1 N HCl, 37°, the pyrophosphate and phosphate esters are slowly hydrolyzed to hydroxymethylpyrimidine.

Purification of Enzyme

Assay—The incubation mixture of 0.5 ml contained the following in micromoles: glycine buffer, pH 9.2, 60; MgSO_4, 0.25; K_2HPO_4, 0.23; and potassium perchlorate, 0.72. The recovery of hydroxymethylpyrimidine-P from a cation exchange column by elution with water is from an unpublished procedure for the synthesis of this pyrimidine compound by Dr. A. L. Morrison, Roche Products, Ltd., London.

The molar ratio of phosphate was determined on the basis of a molar extinction coefficient of 11.2 x 10^4 cm^2 per mole. Hydrolyzable phosphate represents phosphate liberated in 20 minutes by 2 N HCl at 100°. The solvent systems for paper chromatography were: Solvent A, 35% formic acid-acetone (1:1); Solvent B, 50% acetic acid-acetone (1:1); Solvent C, isobutyric acid-1 N NH_4OH (10:6). Chromatograms were run ascending in Solvents A and B and descending in Solvent C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar ratio of phosphate</th>
<th>Solvent system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Hydrolyzable</td>
</tr>
<tr>
<td>Hydroxymethylpyrimidine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxymethylpyrimidine-P</td>
<td>0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>Hydroxymethylpyrimidine-PP</td>
<td>2.0</td>
<td>2.1</td>
</tr>
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</table>

Fig. 1. Acid hydrolysis of ATP (O-O) and hydroxymethylpyrimidine-PP ( ). Aliquots were removed at intervals from tubes immersed in boiling water containing 1.5 μoles of ATP or hydroxymethylpyrimidine-PP in 2 ml of 1 N H_2SO_4. The formation of 2 μoles of orthophosphate per mole of compound is taken as 100% hydrolysis.

mercaptoethanol, 0.5; thiazole-P, 1.0; and hydroxymethylpyrimidine-PP, 1.0. The reaction was initiated by addition of the enzyme suitably diluted in 0.005 M mercaptoethanol. After 5 minutes at 37°, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid, and the thiamine phosphate formed was assayed by the ferricyanide thiocianote procedure. To a 0.1 ml aliquot was added 0.2 ml of 4 M potassium acetate, followed by 0.1 ml of 0.0036 M potassium ferricyanide in 7 N sodium hydroxide and, after mixing, by 0.1 ml of 0.06% hydrogen peroxide in 5.5 M sodium dihydrogen phosphate, and 0.5 ml of water. Thiamine phosphate formation, measured by this procedure or by the cyanogen bromide reaction described under “Methods,” was proportional to enzyme concentration. A unit of activity is defined as that amount of enzyme catalyzing the synthesis of one micromole of thiamine-P at 37° in 5 minutes. Protein was determined by the method of Sutherland et al. (21) or of Warburg and Christian (22).
TABLE II
Purification of thiamine phosphate pyrophosphorylase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>650</td>
<td>101</td>
<td>0.0021</td>
</tr>
<tr>
<td>Heated extract</td>
<td>827</td>
<td>90</td>
<td>0.0043</td>
</tr>
<tr>
<td>Ammonium sulfate I</td>
<td>133</td>
<td>66</td>
<td>0.016</td>
</tr>
<tr>
<td>Protamine extract</td>
<td>54</td>
<td>39</td>
<td>0.18</td>
</tr>
<tr>
<td>Ammonium sulfate II</td>
<td>21.5</td>
<td>29</td>
<td>0.35</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>12</td>
<td>18</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Purification—Anheuser-Busch bakers' yeast, 1.7 kilos, which had been frozen in liquid nitrogen, was thawed and allowed to stand for 18 to 24 hours at 3°. The mixture was centrifuged and the supernatant liquid (crude extract, 650 ml, Table II) was diluted with 0.5 volume of water and brought to 55° in a boiling water bath. After 2 to 3 minutes at 55°, the suspension was quickly cooled to 10° and centrifuged.

The supernatant fluid (crude extract, 650 ml, Table II) was placed on a DEAE-cellulose column (2.8 x 13 cm) which had been packed under a pressure of 8 pounds per square inch and equilibrated with 0.01 M Tris buffer containing 0.005 M mercaptoethanol-EDTA as above, and the active extracts combined (protamine extract).

The enzyme was dialyzed against the same buffer for 24 hours (ammonium sulfate II).

DEAE-cellulose Chromatography—The dialyzed enzyme was placed on a DEAE-cellulose column (2.8 x 13 cm) which had been packed under a pressure of 8 pounds per square inch and equilibrated with 0.01 M Tris-0.005 M mercaptoethanol buffer, pH 7.2. The column was washed with 100 ml of buffer, and the enzyme was recovered by gradient elution at the rate of 5 ml per minute with increasing concentrations of NaCl. The gradient was obtained by allowing 0.5 M NaCl in 0.02 M Tris buffer, pH 7.2, to drop into a mixing chamber containing 500 ml of the same buffer. The enzyme generally appeared after the first 200 ml of eluant in a volume of from 30 to 75 ml. The combined fractions were brought to 70% saturation by the addition of 43.6 g of ammonium sulfate per 100 ml and the precipitate dissolved in 0.04 M Tris-0.005 M mercaptoethanol buffer, pH 7.2 (DEAE).

Properties of Enzyme—The purified enzyme lost less than 10% of its activity when stored for 6 months at -15°, but was completely inactivated when kept at room temperature overnight. Dilutions prepared with 0.1% serum albumin, 0.005 M cysteine or mercaptoethanol are fully active, but large losses are incurred when dilutions are made with quartz-distilled water or with gelatin solution.

pH Optimum—Thiamine and its phosphates exhibit very unusual acid-base properties involving a complex series of equilibria with zwitterion, pseudo-base and thiol forms of the interrelationship of which has been summarized by Metzler (23). Since the average pK of the diprotic transition between thiamine thione and the open ring thiol form is approximately 9.3, it is likely that the observed pH optimum of approximately 9.2 (Fig. 2), may reflect acid-base equilibria of thiamine phosphate rather than of charged groups on the active site of the enzyme.

Metal Requirements—Various preparations were stimulated 3- to 6-fold by 1 x 10^-3 M magnesium. Manganese was approximately half as effective and calcium, zinc, and cobalt were inactive.

Specificity—The enzyme is specific for thiazole-P and hydroxymethylpyrimidine-PP. No reaction occurred when thiazole, or thiazole-PP replaced thiazole-P or when hydroxymethylpyrimidine or hydroxyethylpyrimidine-PP replaced hydroxymethylpyrimidine-PP. The same results were obtained in the presence of ATP, indicating that the corresponding kinase are also absent. About half the enzyme preparations of approximately the same specific activity contained no detectable inorganic pyrophosphatase. Other preparations were variously contaminated with pyrophosphatase up to 10% of the thiamine-P pyrophosphorylase activity.

Stockiometry—The results of balance studies, presented in Table III, are in accord with the reaction expressed in Equation 4. In Experiments 1 to 3, hydroxymethylpyrimidine-PP and PP, were assayed as acid-labile phosphate after having been separated by adsorption of the pyrimidine on 10 to 50 mg of Norit A essentially as described for the analysis of nucleotides by Crane (24). The hydroxymethylpyrimidine-PP, adsorbed on charcoal, was hydrolyzed in 1 ml of 2 N HCl at 100° for 20 minutes. The supernatant was removed, the charcoal was

FIG. 2. Enzyme activity as a function of pH in glycylglycine (Δ) histidine (Ο) and glycine (●) buffers.
washed three times with 0.3 ml of 0.1 N HCl, and the combined supernatant and washings were assayed for phosphate.

Because of the relatively high level of PP, in Experiment 4, a satisfactory estimation of hydroxymethylpyrimidine-PP based on labile phosphate was not achieved. However, by adsorbing thiamine-P and thiazole-P on a cation exchange resin, hydroxymethylpyrimidine-PP could be determined spectrophotometrically. The reaction mixture was deproteinized by the addition of 0.1 ml of 2.5 N HCl and assayed for thiamine-P by the CNBr method for thiamine-P. The enzyme, 7 units. Incubations were performed at 37° for 20 minutes in Experiment 1 to 3 and for 60 minutes in Experiment 4. Thiamine-P was determined fluorimetrically and the other components as described in the text.

### Table III

<table>
<thead>
<tr>
<th>Substance</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Hydroxymethylpyrimidine-PP</td>
<td>-0.48</td>
</tr>
<tr>
<td>Thiamine-P</td>
<td>+0.48</td>
</tr>
<tr>
<td>PP</td>
<td>+0.51</td>
</tr>
</tbody>
</table>

**Effect of Substrates and Inhibition by Pyrophosphate**—Kinetic studies at very low levels of substrate were facilitated by the high sensitivity of the CNBr fluorimetric method for thiamine-P. The concentrations of hydroxymethylpyrimidine-PP and of thiazole-P decreased by approximately 5% during the 5-minute incubation. Figs. 3 and 4 show the effect of each substrate and of PP on the rate of synthesis of thiamine-P. The inhibition by PP is not competitive with either substrate. In similar experiments, increasing the Mg++ ion concentration to 5 x 10^-4 M, approximately 5 times the concentration required to saturate the enzyme, did not overcome the inhibition by PP. The Michaelis constants, estimated from double reciprocal plots (25), were 1 X 10^-8 M for hydroxymethylpyrimidine-PP and 7 x 10^-4 M for thiazole-P.

**Equilibrium**—The equilibrium of the reaction at pH 0.2 was studied in both directions by measuring the change in thiamine-P concentration under conditions favoring synthesis or pyrophosphorylisis (Fig. 5). The addition of inorganic pyrophosphatase, after equilibrium had been reached in the forward direction, caused the reaction to go to completion and indicated that the enzyme was still active. The reverse reaction was studied in the presence of a 5-fold excess of PP. An equilibrium constant for Reaction 4 as written, was determined for eight such experiments; the values ranged from approximately 4.5 to 9 with an average of 6. In favoring synthesis, this reaction resembles the formation of nicotinamide mononucleotide and nicotinamide mononucleotide by similar pyrophosphate displacement reactions (26, 27). In contrast, the synthesis of nicotinamide riboside from ribose-1-phosphate and nicotinamide by phosphate displacement strongly favors pyrophosphorylation (28).

**Enzymatic Assay of Hydroxymethylpyrimidine-PP and Thiazole-P**—In the presence of an excess of one substrate, the limiting component can be quantitatively converted to thiamine-P by using an enzyme preparation containing inorganic pyrophosphatase. The thiamine-P is determined fluorimetrically in the presence of a 5-fold excess of PP, 0.05 µmole; glycine buffer, pH 9.2, 50 µmole; mercaptoethanol, 0.5 µmole; and MgSO4, 0.5 µmole; and approximately 2 X 10^-4 units of enzyme. After 5 minutes at room temperature, the reaction was stopped by the addition of 0.25 ml of 2.5 N HCl and assayed for thiamine-P by the CNBr procedure.
Enzymatic Synthesis of Thiamine Monophosphate

Fig. 5. Reversibility and equilibrium. The reaction mixture (1.0 ml) contained MgSO_4, 0.1 μmole; mercaptoethanol, 0.5 μmole; glycine buffer, pH 9.2, 100 μmoles; and approximately 0.1 unit of enzyme. In pyrophosphorolysis studies, □ and ▲ contained, respectively, 0.074 and 0.044 μmole of thiamine-P and 0.46 and 0.26 μmole of PP_i. In thiamine phosphate synthesis, Δ and ○ contained 0.085 and 0.043 μmole of thiazole-P and 0.1 and 0.05 μmole of hydroxymethylpyrimidine-PP. At the arrows, 5 units (4) of highly purified inorganic pyrophosphatase were added. Aliquots were hydroxymethylpyrimidine-PP. At the arrows, 5 units (4) of highly purified inorganic pyrophosphatase were added. Aliquots were diluted with 0.125 M HCl, and thiamine-P was determined fluorometrically by the CNBr assay.

Fig. 6. The reaction catalyzed by thiamine-P pyrophosphorylase. Abbreviation: PYPP, hydroxymethylpyrimidine-PP.
REFERENCES

The Enzymatic Synthesis of Thiamine Monophosphate
Irwin G. Leder


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